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Prevalence of Tetracycline-Resistant Campylobacter in Organic Broilers During a Production Cycle

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Abstract

Tetracycline (tet) resistance in *Campylobacter* isolated from organically raised broilers was investigated in this study. Two hundred forty-five samples from an organic broiler farm were collected weekly from the first week to the end of the production cycle, and they were cultured for thermophilic *Campylobacter*. Tetracycline resistance of these *Campylobacter* isolates was identified by the agar dilution method, whereas DNA fingerprinting profiles of tet-susceptible and tet-resistant strains were determined by pulsed-field gel electrophoresis (PFGE). None of the *Campylobacter* isolates from the third and the fourth week of the production period were resistant to tetracycline, whereas 66.7% of the isolates from the fifth week were resistant to this antibiotic. Although the prevalence of tetracycline resistance reached 100.0% during the sixth and seventh week, less than 34.0% of the isolates from the 10th week were resistant to this antimicrobial agent. In addition, only 13.8% of *Campylobacter* isolates from the intestinal tracts of these organically raised broilers were resistant to tetracycline. The presence of the *tet*(O) gene was detected in 98.9% of tet-resistant *Campylobacter* isolates, and tet-susceptible and tet-resistant *Campylobacter* strains showed distinct PFGE genotypes. The results suggest that the *Campylobacter* strains isolated from the early stage of the production were susceptible to tetracycline, but they were subsequently displaced by tet-resistant *Campylobacter*.

Keywords

tetracycline resistance, Campylobacter, organic broiler

Disciplines

Genetics and Genomics | Veterinary Microbiology and Immunobiology | Veterinary Pathology and Pathobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

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Research Note—

Prevalence of Tetracycline-Resistant *Campylobacter* in Organic Broilers During a Production Cycle

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SUMMARY. Tetracycline (tet) resistance in *Campylobacter* isolated from organically raised broilers was investigated in this study. Two hundred forty-five samples from an organic broiler farm were collected weekly from the first week to the end of the production cycle, and they were cultured for thermophilic *Campylobacter*. Tetracycline resistance of these *Campylobacter* isolates was identified by the agar dilution method, whereas DNA fingerprinting profiles of tet-susceptible and tet-resistant strains were determined by pulsed-field gel electrophoresis (PFGE). None of the *Campylobacter* isolates from the third and the fourth week of the production period were resistant to tetracycline, whereas 66.7% of the isolates from the fifth week were resistant to this antibiotic. Although the prevalence of tetracycline resistance reached 100.0% during the sixth and seventh week, less than 34.0% of the isolates from the 10th week were resistant to this antimicrobial agent. In addition, only 13.8% of *Campylobacter* isolates from the intestinal tracts of these organically raised broilers were resistant to tetracycline. The presence of the *tet*(O) gene was detected in 98.9% of tet-resistant *Campylobacter* isolates, and tet-susceptible and tet-resistant *Campylobacter* strains showed distinct PFGE genotypes. The results suggest that the *Campylobacter* strains isolated from the early stage of the production were susceptible to tetracycline, but they were subsequently displaced by tet-resistant *Campylobacter*.

RESUMEN. *Nota de Investigación*—Prevalencia de *Campylobacter* resistente a tetraciclina en pollos de engorde orgánicos durante un ciclo de producción.

En este estudio se investigó la resistencia a la tetraciclina en *Campylobacter* aislados de pollos de engorde criados de manera orgánica. Se tomaron semanalmente 245 muestras de una granja orgánica de pollos de engorde desde el inicio hasta el final del ciclo de producción y fueron analizadas para determinar la presencia de *Campylobacter* termofílico. La resistencia a la tetraciclina en estos aislamientos de *Campylobacter* se determinó mediante el método de dilución en agar, mientras que los patrones de restricción del ADN de cepas susceptibles y resistentes a la tetraciclina fueron determinados mediante electroforesis en campo de pulsaciones. Ninguno de los aislamientos de *Campylobacter* del periodo correspondiente a la tercera o cuarta semana de producción resultaron resistentes a la tetraciclina, mientras que el 66.7% de los aislamientos a partir de la quinta semana resultaron resistentes a este antibiótico. Aun cuando la prevalencia de la resistencia a tetraciclina alcanzó un 100% durante la sexta y séptima semana de edad, menos del 34% de los aislamientos de la décima semana eran resistentes a este agente antimicrobiano. Adicionalmente, solo13.8% de los aislamientos de *Campylobacter* provenientes del tracto intestinal de estos pollos de engorde criados de manera orgánica eran resistentes a la tetraciclina. Se detectó la presencia del gen *tet*(O) en 98.9% de los aislamientos de *Campylobacter* resistentes a la tetraciclina y las cepas de *Campylobacter* susceptibles y resistentes a la tetraciclina mostraron patrones diferentes de electroforesis en campo de pulsaciones. Los resultados sugieren que las cepas de *Campylobacter* aisladas en la primera parte del ciclo de producción eran susceptibles a la tetraciclina, pero fueron subsecuentemente desplazadas por *Campylobacter* resistentes a la tetraciclina.

Key words: tetracycline resistance, *Campylobacter*, organic broiler

Abbreviations: PCR = polymerase chain reaction; PFGE = pulsed-field gel electrophoresis; tet = tetracyline

Campylobacter jejuni is one of the leading causes of food-borne bacterial diarrhea in the United States and other countries (1). Most illnesses associated with *Campylobacter* infections are due to consumption of contaminated poultry meats or foods that are cross-contaminated with raw or undercooked poultry (2,10). Because poultry is considered to be a major source of food-borne campylobacteriosis (10,15), development of antimicrobial resistance in *Campylobacter* isolated from poultry is a matter of concern for public health. Over the last decade, the prevalence of tetracycline (tet) resistance in *C. jejuni* and *Campylobacter coli* has increased drastically worldwide (6,8,12,19). Tetracycline is a broad-spectrum antibiotic that has been widely used in human and veterinary medicine since the late 1940s and the early 1950s (4,5,16). The main mechanism of tetracycline resistance in *Campylobacter* species is associated with a ribosomal protection protein designated as Tet(O) (20,21). The Tet(O) protein is encoded by the *tet*(O) gene, which is usually carried on transmissible plasmids (4,8,20,21). Tet-resistant *Campylobacter* is highly prevalent in various animal production systems regardless of history of antimicrobial use (11,18). Because no antibiotics are used in the organic production system, the high prevalence of tet-resistant *Campylobacter* on organic farms cannot be attributable, at least directly, to the presence of antibiotic selection pressure. Although a high prevalence of

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Table 1. Isolation and tetracycline resistance of *Campylobacter* from organic broilers during a production cycle.

Period (wk)	No. of samples positive for *Campylobacter*/no. of samples tested						No. of tet-resistant *Campylobacter*/no. of isolates tested (%)	No. of tet-resistant isolates positive for the *tet*(O) gene (%)
	Feces	Feed	Litter	Grass	Water	Total (%)		
1	0/2	0/3	0/2	0/0	0/2	0/9 (0)	0	0
2	0/2	0/2	0/2	0/6	0/2	0/14 (0)	0	0
3	4/4	1/3	2/4	2/2	2/2	11/15 (73.3)	0/11 (0)	0
4	14/14	1/4	2/4	3/4	4/4	24/30 (80.0)	0/24 (0)	0
5	10/10[A]	2/4[B]	2/4[B]	3/8[B]	4/4[A]	21/30 (70.0)	14/21 (66.7)	13 (92.9)
6	8/8[B]	2/4[B]	4/4[B]	3/4[B]	4/4[B]	21/24 (87.5)	18/18 (100.0)[C]	18 (100.0)
7	14/14[B]	2/2[B]	1/4[B]	3/4[B]	4/4[B]	24/28 (85.7)	24/24 (100.0)	24 (100.0)
8	12/12[A]	0/0	0/2	3/4[A]	3/4[A]	18/22 (81.8)	13/18 (72.2)	13 (100.0)
9	7/14[A]	0/0	0/0	0/0	4/4[A]	11/18 (61.1)	9/11 (81.8)	9 (100.0)
10	17/19[A]	1/2	0/0	0/0	4/4[A]	22/25 (88.0)	7/21 (33.3)[C]	7 (100.0)
Intestine						30/30 (100.0)	4/29 (13.8)[C]	4 (100.0)

[A]Some isolates were resistant to tetracycline.
[B]All isolates were resistant to tetracycline.
[C]Number of *Campylobacter* isolates tested for tetracycline resistance was fewer than the number of *Campylobacter* isolated due to the loss of some of the isolates during storage.

tetracycline resistance in *Campylobacter* isolates from organic chickens has been reported (6,11), little is known about the development dynamics of tet-resistant *Campylobacter* on organic poultry farms. Therefore, the purpose of this study was to investigate the occurrence of tetracycline resistance in *Campylobacter* isolates by following an organic broiler flock for the entire production cycle.

MATERIALS AND METHODS

Sample collection. Fecal and environmental samples from an organic broiler farm located in central Ohio were collected every week from the first week to the end of the production cycle at 74 days of age. Environmental samples including feed, water, litter, and grass as well as fresh fecal samples were collected throughout the farm to ensure that samples represented the whole production system. The number of feces as well as feed, litter, grass, and water samples collected is presented in Table 1. All samples were collected in sterile specimen containers, and they were brought back to the laboratory within 3 hr to culture for *Campylobacter*. In addition, 30 intestinal tracts of these organically raised broilers were also collected at the slaughterhouse.

Isolation of *Campylobacter*. The presence of *Campylobacter* in environmental samples was determined by the selective enrichment method, whereas the presence of this organism in poultry intestines was determined by the direct plating method. For the direct plating method, each sample was directly streaked onto Campy CVA agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD). The inoculated plates were then incubated at 42 C for 48 hr under a microaerophilic environment (approximately 5% O_2, 10% CO_2, and 85% N_2) in a CampyPak II anaerobic system jar with CampyPak gas-generating system envelopes (BBL Becton Dickinson Microbiology Systems). For the selective enrichment method, the procedure was performed according to the protocol published previously (7) with some modifications. Briefly, 10 g of each sample was added to 90 ml of Preston broth (Oxoid Ltd., Basingstoke, Hampshire, England) containing nutrient broth no. 2 (CM0067), *Campylobacter* growth supplement (SR084E/SR0232E), Preston *Campylobacter* selective supplement (SR0117E), and 5% laked horse blood (SR0048C). After each sample was homogenized for 30 sec using a Seward stomacher® lab blender (Brinkman, Westbury, NY), it was incubated at 42 C overnight under a microaerophilic environment as described above. Then, the enriched sample was subcultured onto Campy CVA agar and incubated at 42 C for another 48 hr under a microaerophilic environment. Suspect *Campylobacter* colonies were identified by colony morphological characteristics as well as biochemical characteristics, including Gram stain, oxidase test, catalase test, and *Campylobacter* culture-plate latex agglutination confirmation test (INDX-Campy [jcl]; PanBio INDX, Inc., Baltimore, MD). In addition, the hippurate hydrolysis test was performed to differentiate *C. jejuni* from other *Campylobacter* species.

Antimicrobial susceptibility test. The agar dilution method was used to determine tetracycline resistance of *Campylobacter* isolates in this study as described previously (11). Briefly, *Campylobacter* isolates grown on blood agar plates for at least 24 hr were inoculated into Mueller-Hinton broth, and then the suspensions were adjusted to a turbidity equivalent to 0.5 McFarland standard. Approximately 10^4 colony-forming units of these suspensions were applied onto Mueller-Hinton agar containing a twofold dilution series (0.06–128 μg/ml) of tetracycline (Sigma Chemical Co., St. Louis, MO) and supplemented with 5% defibrinated sheep blood using a multipoint inoculator with 1-mm pins (Oxoid, Inc., Ogdensburg, NY). *Campylobacter jejuni* ATCC 33560 was also inoculated onto each plate to serve as a quality control organism. The inoculated plates were incubated at 42 C under a microaerophilic condition for 24 hr. The Clinical and Laboratory Standards Institute's (formerly National Committee on Clinical Laboratory Standards)-recommended breakpoint for *Enterobacteriaceae* was used to determine tetracycline resistance of *Campylobacter* isolates in this study (13). If the isolate had the minimal inhibitory concentration $\geq$ 16 μg/ml, it was classified as tet-resistant.

Polymerase chain reaction (PCR). The presence of the *tet*(O) gene was determined by PCR according to the protocol published previously (3). Briefly, PCR was performed in a volume of 50 μl with a final concentration of each component as follows: 1× PCR buffer, 2.5 mM $MgSO_4$, 0.2 mM each deoxynucleoside triphosphates, 0.2 μM primers, and 1.25 U of *Taq* DNA polymerase. Primers used for the *tet*(O) gene detection in this study were TetO-FW (5′-ACG GAR AGT TTA TTG TAT ACC-3′) and TetO-RV (5′-TGG CGT ATC TAT AAT GTT GAC-3′), which produced a PCR product of 171 base pairs. A GeneAmp® 2400 PCR system (PerkinElmer Corporation, Norwalk, CT) was used for PCR amplification, with an initial denaturation step at 94 C for 5 min; followed by 25 cycles of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 30 sec; and then a final extension step at 72 C for 10 min.

Pulsed-field gel electrophoresis (PFGE). To determine genomic DNA fingerprinting profiles of *Campylobacter* isolates, PFGE was performed using the protocol described previously (9). Briefly, fresh *Campylobacter* cells were washed with phosphate-buffered saline and then adjusted to an optical density of 0.65 at a wavelength of 610 nm by a spectrophotometer. Approximately 400 μl of cell suspension was mixed with 20 μl of proteinase K (20 mg/ml) and incubated at 50 C. To prepare bacterial DNA plugs, an equal amount of melted 1% SeaKem Gold agarose was added to the cell suspension and mixed gently. The agarose plugs containing whole bacterial cells were allowed to solidify at room temperature for 10–15 min, and then they were lysed

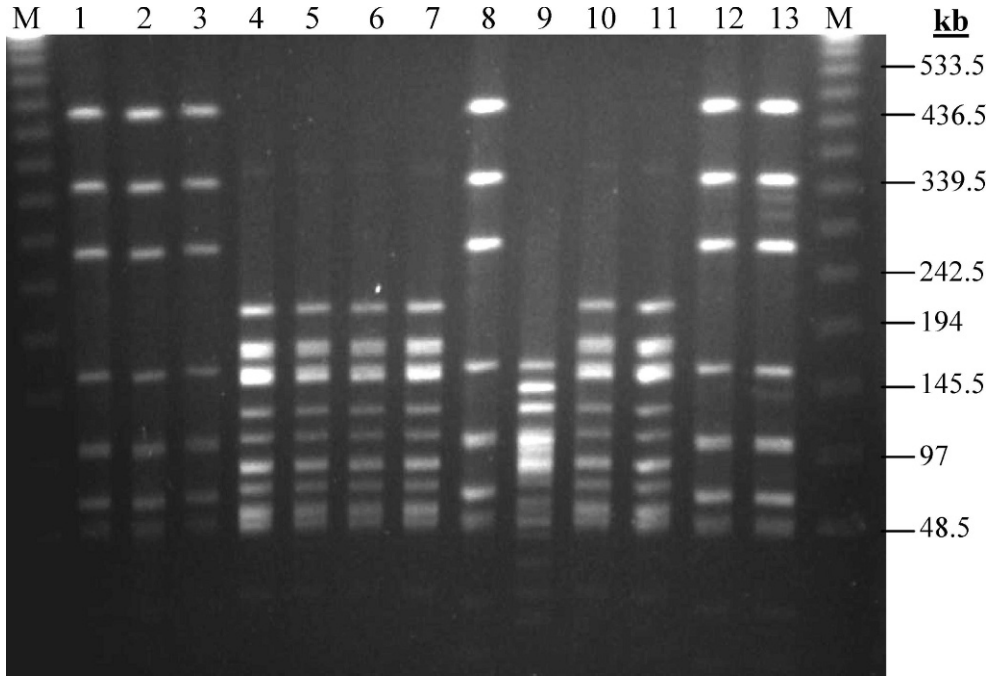

Fig. 1. PFGE patterns of representative tet-susceptible and tet-resistant *Campylobacter* strains isolated from organically raised broilers. Lanes 1, 2, and 3 are tet-susceptible strains from week 3, 4, and 5; lanes 4, 5, 6, and 7 are tet-resistant strains from week 5, 6, 7, and 8; lanes 8 and 9 are tet-susceptible strains from week 8 and 9; lanes 10 and 11 are tet-resistant strains from week 9 and 10; and lanes 12 and 13 are tet-susceptible strains from week 10 and intestine. Molecular weight markers (Bio-Rad® Lambda DNA ladder) are shown in lanes M. All tet-resistant isolates share a single PFGE type. The genomic DNA was digested with KpnI before electrophoresis.

in lysis buffer with proteinase K (at the final concentration of 0.1 mg/ml) at 52 C in a shaking water bath for 30 min. After lysis, the agarose plugs were washed with reagent grade water twice and then washed with Tris-EDTA buffer three times. The agarose plugs were digested with KpnI restriction enzyme at 37 C for 5 hr. The digested plugs were embedded into 1% SeaKem Gold agarose gel. Electrophoresis was performed using a CHEF Mapper XA Pulsed Field Electrophoresis system (Bio-Rad, Hercules, CA). The electrophoresis was run for 18 hr. After the run was completed, the gel was stained with ethidium bromide for 25 min, and then it was washed three times with reagent grade water. The genomic DNA fingerprinting profiles were visualized with UV transilluminator and photographed with a digital imaging system (Alpha Innotech, San Leandro, CA).

RESULTS

***Campylobacter* isolation.** Thermophilic *Campylobacter* was not recovered from any environmental or fecal samples collected during the first and second week of the production period (Table 1). During the third to the 10th week of the organic broiler production cycle, the percentage of samples positive for *Campylobacter* varied from week to week, ranging from 61.1% to 88.0% (Table 1). At the end of the production cycle, *Campylobacter* was isolated from all 30 intestinal tracts (100.0%) of these organic broilers (Table 1). All *Campylobacter* isolates from this organic broiler farm were *C. jejuni* on the basis of hippurate hydrolysis test.

Tetracycline resistance determination. None of the *Campylobacter* isolates from the third and fourth week of the production cycle were resistant to tetracycline, whereas 66.7% of the isolates from the fifth week were resistant to this antibiotic (Table 1). Prevalence of tetracycline resistance reached 100% during the sixth and seventh week, and then it reduced to 72.2% and 81.8% in the eighth and ninth week of the production cycle, respectively (Table 1). At week 10, about 33.3% of these *Campylobacter* isolates were resistant to tetracycline, whereas only 13.8% of *Campylobacter* isolates from the intestinal tracts of the organically raised broilers were resistant to this antimicrobial agent (Table 1). In terms of the association between sample source and tet-resistant *Campylobacter* isolates, we found that all isolates from feed, litter, and grass samples collected during week 5 to 7 were resistant to tetracycline, whereas some isolates from water and fecal samples were resistant to this antibiotic (Table 1). Although more than 60% of *Campylobacter* isolates from fecal samples collected during the fifth to the ninth week were resistant to tetracycline, only 23.5% (4/17) of the isolates from fecal samples collected at week 10 were tet-resistant strains. All tet-resistant *Campylobacter* isolates except one from the fifth week of the production cycle were positive for the *tet*(O) gene (Table 1), whereas none of the tested tet-susceptible *Campylobacter* isolates was positive for this gene.

DNA fingerprinting profiles. Two major PFGE patterns were dominant on this organic broiler farm. One PFGE pattern was for tet-susceptible strains and the other pattern was for tet-resistant strains (Fig. 1). Multiple PFGE experiments were performed, and the PFGE patterns were consistently shown in multiple gels.

DISCUSSION

Lack of *Campylobacter* in the broilers for the first 2 wk was consistent with the findings from previous studies (14,17), which also revealed that *Campylobacter* species are rarely detected in broiler flocks under the age of 2–3 weeks old. The explanation for this phenomenon is unclear; however, it is possible that the presence of *Campylobacter*-specific maternal antibodies in young chicks or the presence of unique microbial flora in the intestinal tract of the chicks may play a role in inhibiting *Campylobacter* colonization during the first 2 weeks of life of the birds (14,17).

Although the explanation for the differences in tetracycline resistance prevalence during the production cycle of organic broilers was inconclusive at this stage, the high resistance prevalence observed during the fifth to the ninth week of the production cycle might be due to the invasion of the organic broiler farm by tet-resistant strain, which quickly colonized the birds and displaced tet-susceptible strain predominant in the intestinal tract of the bird. This explanation is supported by the PFGE patterns of the isolates, which showed distinct PFGE types between tet-resistant and tet-susceptible strains. The obvious differences in the PFGE patterns between tet-resistant and tet-susceptible strains indicate that they are genetically distinct clones and suggest that tet-resistant *Campylobacter* strains detected in the fifth week was not developed from the preexisting tet-susceptible strain by acquisition of the *tet*(O) gene. Because organic broilers were reared on the pasture and exposed to the outside environment most of their lives, it is possible that these birds may pick up tet-resistant *Campylobacter* strain from the farm environment. Interestingly, tet-susceptible strain that was dominant during the third and fourth week as well as a new tet-susceptible PFGE type was detected in the late stage of the production (Fig. 1, lanes 9, 12, and 13). These observations suggest that the organic broiler farm is vulnerable for invasion by different *Campylobacter* strains with or without tetracycline resistance.

In summary, this study reveals the complex and dynamic nature of tetracycline resistance in *Campylobacter* isolates on the organic poultry farm. The results suggest that the early *Campylobacter* strains isolated from this organic broiler farm were susceptible to tetracycline, but later were replaced by a tet-resistant strain. Although the invasion of poultry flock by tet-resistant *Campylobacter* strain was responsible for the occurrence and the prevalence of tetracycline resistance on this organic broiler farm, it was not clear how this tet-resistant *Campylobacter* strain was introduced into this farm. However, it is clear that tet-resistant *Campylobacter* can transmit and persist even in the absence of antibiotic selection pressure. Further studies are required to determine the effect of tetracycline resistance on the fitness and transmission of *Campylobacter* in poultry reservoirs.

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